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Efficacy of Porcine Gonadotropins for Repeated Stimulation of Ovarian Activity for Oocyte Retrieval and In Vitro Embryo Production and Cryopreservation in Siberian Tigers (*Panthera tigris altaica*)[1]

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ABSTRACT

A comparison of the amino acid sequences demonstrated that Siberian tiger gonadotropins are more homologous with those of porcine than any other commercially available preparation. The present study measured the efficacy of repeated ovarian stimulation with purified porcine gonadotropins on the follicular, hormonal, and immunogenic responses in Siberian tigers as well as on the ability of oocytes retrieved by laparoscopic follicular aspiration to fertilize and cleave in vitro. Controlled rate and vitrification cryopreservation methods were also compared for their ability to support ongoing cleavage following thawing of presumptive 2- to 4-cell tiger embryos generated in vitro. Vitrification supported continued embryonic cleavage in vitro while controlled rate freezing did not. Stereological microscopy indicated an excellent ovarian response with the recovery of quality cumulus-oocyte complexes that apparently fertilized and cleaved in vitro. However, ultrastructural and physiological examination revealed abnormal and unnatural responses such as the failure of some cumulus-oocyte complexes to reach maturity and progestagen levels to approach normalcy. At the same time, analyses of blood for antibodies failed to detect an immune reaction to these foreign gonadotropins in an assay that tested positive for the chorionic gonadotropin-stimulated domestic cat. Together, these observations suggest that porcine gonadotropins may be effective for the ovarian stimulation of tigers but that some modifications to administration protocols are needed to produce a more natural response.

assisted reproductive technology, follicle-stimulating hormone, luteinizing hormone, ovary

INTRODUCTION

Reproductive technology is becoming an increasingly important tool in the genetic management and breeding of captive populations of many endangered species [1, 2], especially in light of mounting restrictions on the movement of animals by animal health regulatory agencies. Designing an appropriate gonadotropin regimen to manipulate ovarian activity, synchronize estrous cycles of recipients with those of embryo donors, and induce hormonal conditions conducive to sustaining pregnancy are difficult obstacles to overcome in assisted reproduction. This is particularly true in species such as felids for which there is no readily available homologous gonadotropin source.

Previous attempts to stimulate ovarian activity in nondomestic cats have followed protocols developed in the domestic cat [3–6]. Several studies focused on varying the dose and timing of administration of eCG to promote follicular growth and hCG to induce follicle and oocyte maturation, ovulation, and luteal function [7–10]. Such regimens have been successful in stimulating ovarian activity in a number of endangered felids followed by the production of embryos in vitro. Furthermore, offspring have resulted from either artificial insemination (see Table 61-7 in [11]) or in vitro fertilization and intra- or interspecies embryo transfer from the chorionic gonadotropin-stimulated Siberian tiger [9, 12], puma [13, 14], leopard cat [15, 16], clouded leopard [17], snow leopard [18], Persian leopard [19], ocelot [20], caracal, and cheetah [7, 21, 22]. Overall, however, pregnancy and offspring survival rates have been low, litters have been small, and failures have been frequent.

The chorionic hormones are often employed because they have long half-lives (24–48 h), alleviating the need for potentially stressful, repeated stimulation (injection) to sustain hormone levels. However, persistent circulating gonadotropins can result in ovarian hyperstimulation and ancillary follicle development [9, 23–26]. Furthermore, it is thought that these chorionic hormones elicit abnormal endocrine events that compromise tubal transport, fertiliza-

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tion, and implantation [27, 28]. Finally, these foreign gonadotropins have been found to induce an immunogenic reaction in several species, including felids [25, 29], goats [30], cows [31], rabbits [32], and nonhuman primates [33–35], thereby decreasing ovarian responsiveness over time. A critical aspect of such studies is the selection of an appropriate dose as well as the interval between successive stimulations in order to maximize the yield and minimize the problem of an immune response.

Other attempts to stimulate ovarian activity in cats have utilized crude (variably contaminated with LH) commercial preparations of porcine FSH (FSH-P), a gonadotropin that has a considerably shorter half-life (about 2 h) than the chorionic hormones. These efforts have met with variable success [4, 36–38], differences in the ovarian response probably resulting from stress produced by the need for repeated injections or the lack of homology of the stimulating agent. As with the chorionic hormones, foreign proteins in an unpurified hormone preparation may increase the likelihood of humoral rejection of the stimulating agent. In utilizing porcine gonadotropin to stimulate follicular activity in a variety of nondomestic cats, the variable success rate has been attributed to the unknown status of ovarian development prior to the start of treatment [39]. Excessive doses of gonadotropin may also have contributed to the variable data obtained from such experiments.

Within the confines of species variation in sensitivity to exogenous hormone treatment, a revised gonadotropin regimen is an obvious area to target in our efforts to better assist reproduction in endangered felids. The tiger is a particularly good model for such research because in vitro fertilization (IVF) generates a high degree of success in this taxonomic group of endangered felids [9, 40]. This may be related to the better quality of tiger ejaculates compared with other endangered large cat species [9, 40–42]. In addition, females are very responsive to exogenous gonadotropin treatment, usually producing large numbers of oocytes. With this objective, we determined that the primary amino acid sequences of the common glycoprotein hormone α subunit and the respective β subunits of tiger FSH and LH more closely resemble porcine than any other commercially available preparations. Pope et al. [43–48] have previously reported on the successful induction of an ovarian response with purified porcine gonadotropins in a number of large cat species, including the tiger, as well as the preparation of domestic cat recipients for the successful inter- and intraspecies transfer of embryos. However, no previous studies have examined the physiological response to exogenous porcine hormone stimulation in detail.

The objective of this study was to determine the efficacy of repeated treatments of purified pFSH and pLH (Sioux Biochemical) on the ovarian response and in vivo maturation of Siberian tiger oocytes and subsequent embryonic development (cleavage) following IVF. A second objective was to establish whether the steroid metabolite and circulating antibody responses following ovarian stimulation were adversely affected by our protocol. Finally, we compared the effects of rate of freezing (controlled slow versus vitrification) on the efficiency of in vitro-produced tiger embryo cryopreservation.

MATERIALS and METHODS

RNA Isolation

A *Panthera tigris altaica* anterior pituitary gland was obtained from the Henry Doorly Zoo and frozen in liquid nitrogen. Subsequently, the tissue and cells were disrupted with TRIzol reagent (Life Technologies, Grand Island, NY) using a polytron homogenizer (Brinkmann Instruments, Westbury, NY). Total RNA was isolated from 50 mg tissue homogenized in 1 ml TRIzol. Chloroform (200 μl) was added and samples were shaken for 15 sec (12 000 × *g*), incubated at 21°C for 3 min and then centrifuged at 1200 × *g* for 15 min. The clear, aqueous phase was transferred into 0.5 ml sterile isopropanol and incubated at 21°C for 10 min, then centrifuged for 10 min. The alcohol was removed from the pellet and the RNA was washed once with 1 ml sterile 75% ethanol. The pellet was allowed to air-dry for approximately 5 min and was then resuspended in 50–100 μl diethyl pyrocarbonate-treated water.

Reverse Transcription Polymerase Chain Reaction

The total RNA was quantified by measuring absorption at 260/280 nm ratios. The methods of 3′ and 5′ random amplification of cDNA ends (RACE) were used to obtain the entire coding sequence of the genes of interest: glycoprotein hormone alpha, follicle stimulating hormone beta (FSHβ), and luteinizing hormone beta (LHβ). The first strand complementary DNA (cDNA) was synthesized from 1 μg total RNA isolated from the anterior pituitary tissue with Superscript II (Life Technologies) at a final concentration of 2.5 U in the Life Technologies first strand buffer containing 25 mM Tris-HCl (pH 8.3), 37.5 mM KCl, 4 mM $MgCl_2$, 4 mM dithiothreitol, 0.5 mM of each deoxy-NTP, and 2.5 μm of oligo dT primer or random hexamers. Incubation was performed at 42°C for 50 min, followed by 70°C for 14 min. RNA was then digested with ribonuclease H (Life Technologies) at 37°C for 20 min. Reactions used for 5′ RACE were tailed with dCTP according to manufacturer specifications (Life Technologies).

The polymerase chain reaction (PCR) mixture totaled 50 μl and consisted of 5 μl of the first strand reaction product, 2.5 U Taq polymerase (Life Technologies), and PCR buffer solution (Life Technologies) containing 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 1 mM $MgCl_2$; 0.2 mM of each deoxy-NTP; 10 pmol of a gene specific oligonucleotide to the α, FSHβ, or LHβ gene; and 10 pmol of the appropriate universal adapter primer (Life Technologies). Oligonucleotides for PCR were designed in consensus sequence regions of the signal peptide and the 3′ ends of the genes based on DNA sequence alignments for several species. Oligonucleotide primers were synthesized by the Eppley Institute Molecular Biology Core Laboratory on an Applied Biosystems (Foster City, CA) model 394 DNA/RNA synthesizer.

Fifteen microliters of the reverse transcription-PCR (RT-PCR) products with 7.5 μl of methyl green/yellow food coloring:50% glycerol were electrophoresed on a 1% (wt/vol) agarose gel in Tris-glacial acetic acid-EDTA buffer. The gel contained 0.2 μg/ml ethidium bromide to visualize the amplified products. RT-PCR products were sequenced by the Eppley Institute Core Laboratory on a 373 ABI sequencing apparatus.

Animals

Four adult Siberian tiger females (ages 5–11 yr) were delegated to this study, which covered a time span of 11 mo. Phase 1 (n = 2) was conducted between February 1998 and September 1998 and phase 2 (n = 2) between June 1998 and January 1999. These animals were housed indoors overnight with free access to the outdoors during daylight hours. They were provided a carnivore diet (Nebraska Brand Feline Diet, North Platte, NE) and water ad libitum.

Ovarian Stimulation

In order to stimulate follicular growth and oocyte maturation (oocyte donors), tigers were treated repeatedly (three consecutive stimulations [trials] per animal ≥100 days apart) with 50 U purified (of any contaminating LH) pFSH (Sioux Biochemical, Sioux Center, IA) administered i.m. by blow dart in a decreasing regimen over 3 days as follows:

Day 1: 20 U pFSH at 0200 h and 15 U at 1600 h.
Day 2: 10 U pFSH at 1600 h.
Day 3: 5 U pFSH at 1600 h.

The following morning (80 h following the first pFSH), 25 U pLH (Sioux Biochemical) were administered.

This protocol was chosen after Donoghue et al. [23], Goodrowe et al. [37], and Pope et al. [43].

Laparoscopic Visualization of Ovaries and Recovery of Oocytes

Twenty-four to 28 h after pLH, anesthesia was induced with i.m. xylazine (Rompun, Mobay Co., Shawnee, KS; 0.5 mg/kg), diazepam (Valium, Hoffman La Roche, Nutely, NJ; 0.1 mg/kg), and ketamine hydrochloride (Vetalar, Park-Davis, Detroit, MI; 5.0 mg/kg) and was sustained by intubation with halothane gas/oxygen. Tigers were placed in a supine position and a pneumoperitoneum created with CO_2 infused via a transabdominally placed Verres needle. Laparoscopic surgery was performed using a 10-mm, 180-degree laparoscope (Richard Wolf Medical Instruments Co., Rosemont, IL) inserted through a 2-cm skin incision near the umbilicus while the ovaries were held in a fixed position by a transabdominally inserted Verres needle probe. All ovarian structures were counted and measured and those follicles ≥2 mm were aspirated with a 19-gauge needle using a Cook vacuum pump (Cook Veterinary Products, Eight Mile Plains, QLD, Australia). Tubes containing the aspirate were quickly transferred to a 37°C waterbath. As soon as possible, the contents were moved to search dishes containing prewarmed TL-Hepes medium (BioWhittaker, Walkersville, MD) containing 10 μg/ml heparin and examined under a stereomicroscope for expanded cumulus-oocyte complexes (COCs).

In Vitro Fertilization and Culture of Embryos

COCs were transferred to fresh, warm TL-Hepes, graded according to numbers of layers of investing cumulus cells and color and uniformity of cytoplasm, and then moved into 50-μl droplets of fertilization medium (modified Tyrode + 6 mg/ml BSA) [49] under mineral oil in Nunc wells (Fisher, Pittsburgh, PA). COCs were coincubated overnight (38°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2) with frozen-thawed [50] Siberian tiger sperm ($\sim 1 \times 10^6$ sperm/ml concentrated through a Percoll density gradient centrifugation at $700 \times g$ for 30 min). The following morning, presumptive zygotes were washed (four times) in TL-Hepes, transferred to culture medium (Tyrode + 3 mg/ml BSA and nonessential amino acids) [49], and returned to the incubator for 24–32 h. Embryo cultures were then examined (40–50 h postinsemination) to determine the percentage of oocytes that had undergone cleavage. At this time, all presumptive embryos (2- to 4-cell stage) were cryopreserved.

Oocyte and Embryo Morphology

Randomly selected samples (N = 25) of expanded COCs within 2 h of retrieval (pre-IVF), presumptive zygotes post-IVF (18–26 h post-insemination) (IVF), and cleaved embryos (IVC) were rinsed in 0.1 M phosphate buffer, fixed in cold 3% glutaraldehyde in 0.1 M phosphate buffer for 1 h at 4°C, then transferred to cold phosphate buffer and stored in 0.5-ml straws for a variable period of time (1–7 mo) before shipment to Copenhagen, Denmark. After receipt, COCs and embryos were prepared for light microscopy (LM) and electron microscopy (TEM) as previously described [51]. Semithin sections were stained with toluidine blue and examined by LM, and selected sections were re-embedded and prepared for ultrathin sectioning. These sections were contrasted with uranyl acetate and lead citrate and examined on a Phillips CM 100 transmission electron microscope.

Fecal Collection, Extraction, and Analysis

Fecal samples were collected 3–7 times/wk from two animals for 1 mo prior to and for 2 mo following each ovarian stimulation, and fecal steroid metabolites were extracted according to Brown et al. [52]. In brief, 0.2 g of lyophilized feces were extracted twice with 5 ml of 90% ethanol:distilled water, the supernatants were combined, dried completely, and redissolved in 1 ml methanol. Extracted samples were diluted in PBS (0.02 M NaH_2PO_4, 0.03 M Na_2HPO_4, 0.14 M NaCl, 0.01% sodium azide, pH 7) before further analysis. The fecal steroid metabolite concentrations were quantified using radioimmunoassays (RIA) previously validated for tigers [27]. Fecal estradiol concentrations were measured by RIA employing an antibody raised in rabbits against estradiol 17-β-6-o-carboxymethyloxime BSA (provided by Dr. Samuel Wasser, Center for Wildlife Conservation, Seattle, WA). Fecal progestagens were measured using an RIA employing a monoclonal progesterone antibody produced against 4-P-11-ol-3, 20-dione hemisuccinate:BSA (Quidel clone #425, provided by Dr. Jan Roser, University of California, Davis, CA). Assay sensitivities were 2 and 5 pg/tube for the estrogen and progestagen assays, respectively, and intra- and interassay coefficients of variation were <10%.

The results obtained from each tiger were combined and a mean and standard error (±SEM)/wk were obtained. A standard *t*-test was used to compare weekly averages between trials.

Blood Collection and Testing for Antibodies

To test for the presence of neutralizing immunoglobulins against the repeated use of porcine hormones, blood was drawn from each tiger before treatment as well as at each time of oocyte collection for the three stimulations. Plasma and serum were frozen (−86°C) until analysis. Sera from three domestic cats that had been stimulated to ovulate with exogenous chorionic gonadotropins were also evaluated concurrently with tiger samples.

Specific binding of tiger gamma globulin to pFSH or pLH was assessed by incubation of serum with iodinated preparations of these two hormones followed by separation of bound from unbound hormone by precipitation with anti-feline gamma globulin. The pFSH (AFP-1064OB) and pLH (AFP-10714B) were iodinated (I-125) by the Chloramine-T method [53]. Tiger serum (4 μl of from each collection), normal (nonimmunized) cat serum (to estimate nonspecific binding), or immunized cat serum (n =3) (control) were incubated in multiple tubes overnight at 4°C with either of the iodinated hormones. This was followed by addition of varying volumes (6–12 μl) of goat anti-feline serum (ICN/Cappel), 24 h of incubation at 4°C, then addition of 1.5 ml of 10% polyethylene glycol 8000. Tubes were incubated for an additional 2 h at 4°C, centrifuged, and the supernatant decanted. The amount of precipitated radioactivity in each tube was determined with a gamma counter. Immunoreactivity of iodinated hormones was verified by displacement with unlabeled porcine hormone in standard RIAs that used antisera prepared in rabbits [54]. Specificity was also confirmed in a sample from an immunized cat by displacement with nonradioactive pLH (300 ng). In addition, tiger sera were evaluated against nonradioactive pFSH and pLH in Ouchterloney immunodiffusion plates.

Freezing, Thawing, and Culture of Tiger Embryos

Embryos (2- to 4-cell, ~40–50 h postinsemination) were cryopreserved according to one of three protocols as follows.

1. *Controlled rate 1.* Embryos (n = 55) were equilibrated (15 min) in 1.4 M propylene glycol (PG) and 0.125 M sucrose (S) in TL-Hepes plus 10% fetal calf serum (Hyclone, Logan, UT) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin; Life Technologies). They were then loaded into 0.25-ml straws (IMV International City) and placed in a programmable FTS unit at room temperature. They were cooled at 2°C/min to −6°C and held for 15 min prior to seeding. After 10 min, they were cooled at 0.3°C/min to −30°C and plunged into liquid nitrogen. For thawing, straws were held in air (8 sec), swirled in a 32°C waterbath and the embryos released. Embryos were rehydrated in three 5-min steps in decreasing concentrations of PG (0.94 M, 0.45 M, and 0) plus S (0.125 M) at room temperature [55].

2. *Controlled rate 2 (direct transfer method).* Embryos (n = 21) were equilibrated (5–10 min) in 1.5 M ethylene glycol (EG) in TL-Hepes medium, loaded into 0.25-ml straws, placed in a FTS unit at −7°C, and seeded. After 10 min, the embryos were cooled at 0.5°C/min to −30°C and plunged into liquid nitrogen. For thawing, straws were warmed as above, and the liberated embryos were rehydrated directly into cryoprotectant-free TL-Hepes medium (containing 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin) at room temperature (for reviews, see [56, 57]).

3. *Vitrification.* Embryos (n = 70) were first equilibrated at room temperature in 1 M DMSO plus 1.35 M EG in TL-Hepes (3 min), then in 2.3 M DMSO plus 3 M EG and 0.5 M S (20 sec), during which time they were loaded into open pulled straws (OPS; DEMTEK, Aarhus, Denmark) in a total volume of 2–5 μl. They were then plunged into liquid nitrogen and stored in labeled polypropylene tubes. For thawing (warming), embryos were released from OPS straws directly into TL-Hepes (room temperature) containing decreasing concentrations of S in three 5-min steps [58].

Following warming, all embryos were placed on Buffalo rat liver cell monolayers that had been conditioned for 24 h with Menezo B2 Medium (Laboratoire CCD, Paris, France) plus 10% fetal calf serum. They were then incubated in a humidified atmosphere (38°C, 5% CO_2 in air) for 24 h and examined for cleavage status. The results of these experiments were compared with the in vitro cleavage rates of tiger embryos (n = 300) prior to freezing/vitrification procedures.

LH-β:

^{1}SRGPLRPLCR^{10}PINATLAAEN^{20}EACPVCVTFT^{30}TTICAGYCPS^{40}MMRVLPAALP^{50}
I S V

PVPQPVCTYR^{60}ELRFASVRLP^{70}GCPPGVDPVV^{80}SFPVALSCRC^{90}GPCRLSSSDC^{100}
S I T H

GGPRAQPLAC^{110}DRPPLPGLPF^{120}L
P P

FSH-β:

^{1}KSCELTNITI^{10} TVEKEECRFC^{20} MSINATWCAG^{30}YCYTRDLVYK^{40}
N I T

DPARPNNQKT^{50}CTFKELVYET^{60}VKVPGCAHQA^{70}DSLYTYPVAT^{80}
A H

ECHCGKCDSD^{90}STDCTVQGLG^{100}PSYCSFSEMK^{110}E
R

common α-subunit:

^{1}FPDGEFTMQG^{10}CPECKLKENK^{20} YFSKLGAPVY^{30}QCMGCCFSRA^{40}
I

YPTPARSKKT^{50} MLVPKNITSE^{60} ATCCVAKAFT^{70}KATVMGNAKV^{80}
R

ENHTECHCST^{90}CYYH

FIG. 1. The comparative sequences of tiger and porcine gonadotropin subunits. Shown above are the amino acid sequences for tiger LHβ, FSHβ, and the common glycoprotein α subunit (GenBank accession numbers AF354938, AF354937, and AF354939, respectively). Also shown (below and in bold) are the porcine amino acid residues that differ from those of the tiger in each corresponding subunit.

RESULTS

DNA Sequences

The DNA sequences of Siberian tiger FSHβ, LHβ, and the common α subunit were determined and validated as described in the *Materials and Methods* and were submitted to the GenBank database (accession numbers AF354937–9, respectively). Primary amino acid sequences that were derived from the nucleotide sequences were aligned and compared with those of human, bovine, porcine, and ovine, species whose FSH and LH are commercially available and could potentially be used for IVF in tigers. The tiger sequences depicted in Figure 1 highlight differences with the porcine, with which the tiger gonadotropins showed the closest homology. The common α DNA sequence was 98% homologous with the porcine α subunit, and tiger FSHβ and LHβ were 95% and 93% homologous to the respective porcine subunits. Based on this high degree of sequence homology, pFSH and pLH were chosen for tiger ovarian stimulation.

Ovarian Response, Oocyte Production In Vivo, and In Vitro Embryo Production

Tigers that were prepared for oocyte retrieval responded to hormonal stimulation with multiple follicle production (average = 33/female; range 17–54). Under laparoscopic examination, follicles were clear and not raised from the ovarian surface (Fig. 2). The majority of retrieved oocytes from all three stimulations were surrounded by a mass of expanded (mucified) cumulus cells (Fig. 3); these were presumed to be mature and were classified as good to excellent in quality [59]. The remainder were contained within only a few layers of tightly compact cumulus cells and were likely immature. No degenerate oocytes were observed. The number of follicles visualized (≥2 mm), presumptively immature and mature COCs retrieved, and the percent presumed to be fertilized (= cleaved)/trial for four tigers are presented in Table 1. Overall, an estimated minimum average of 45% (trial 1), 62% (trial 2), and 52% (trial 3) of presumptive zygotes cleaved.

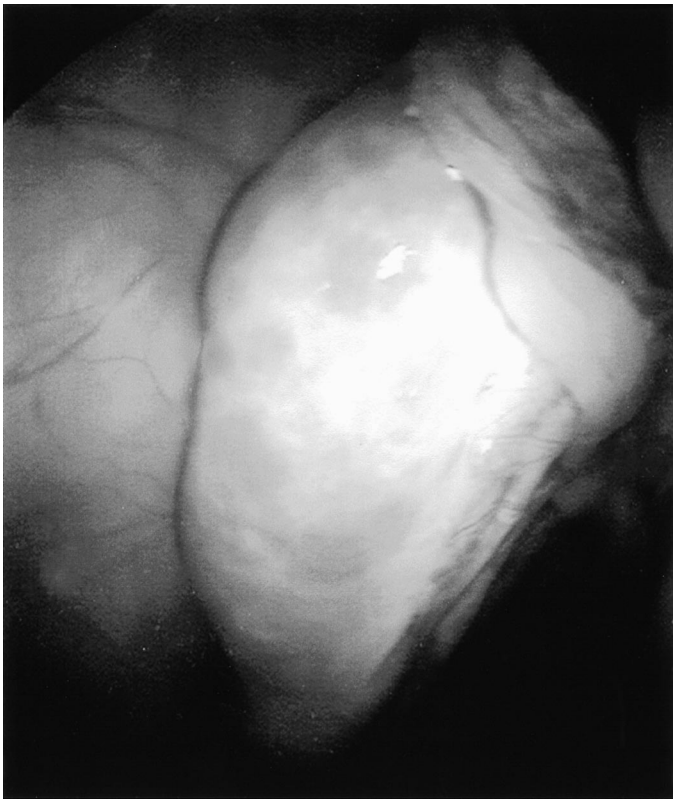

FIG. 2. Laparoscopic view of ovarian surface features in a tiger stimulated with porcine gonadotropins.

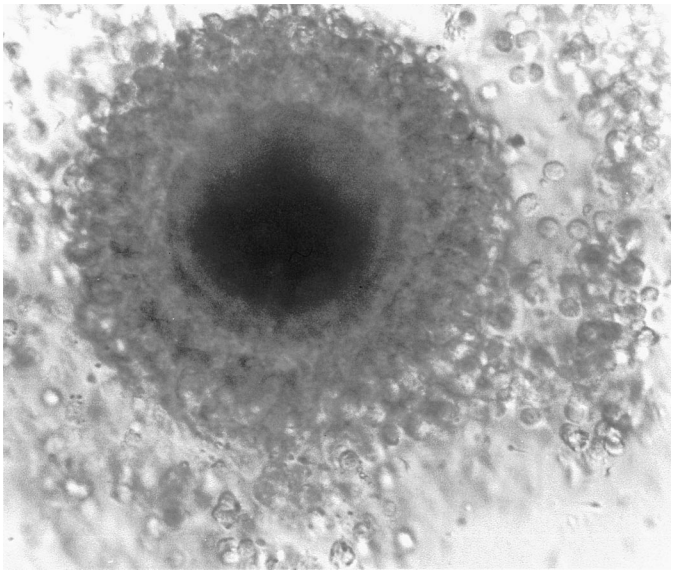

FIG. 3. Cumulus-oocyte complex recovered by laparoscopic follicular aspiration in a tiger stimulated with porcine gonadotropins.

TABLE 1. Numbers of ovarian follicles, presumptively immature and mature oocytes, and cleavage rate (percent fertilized) observed in each of four tigers stimulated three times with porcine gonadotropins.

	Trial											
	1	2	3	1	2	3	1	2	3	1	2	3
Follicles	17	26	29	36	X*	33	34	45	54	29	29	34
Immature oocytes	2	7	5	4	X*	4	11	6	6	3	6	1
Mature oocytes	7	33	24	30	X*	14	39	41	56	26	26	33
% Fertilized	50	73	60	71	X*	50	36	62	65	25	50	35

* Respiratory problems during anesthesia—experiment aborted.

Together, these data imply that stimulation with porcine gonadotropins elicited a satisfactory ovarian response in tigers prepared for oocyte retrieval following three consecutive trials.

Oocyte and Embryo Morphology

Gross stereomicroscopic analysis of COCs presented a normal morphology as defined by similar observations on domestic cat oocytes in vitro [60]. The ooplasm in most of the oocytes was dark and finely granulated while the surrounding cumulus cells were light (Fig. 3). In 2- and 4-cell embryos, cleavage was symmetrical and the ooplasm was dark and vacuolated (Fig. 4).

LM and TEM examination revealed a number of ultrastructural features inherent to oocyte maturation, fertilization, and early embryonic development. A number of maturational and developmental deviations were noted, however. These included a broad range of different maturational stages in COCs classified as presumptively mature. Further, indications of both cytoplasmic and nuclear fragmentation could be observed in early cleaved embryos, resulting in polynucleated blastomeres (Fig. 5), while cell fragments were observed in the perivitelline space. Table 2 summarizes the abnormalities seen by presenting the stage of maturation, fertilization, or development reached by oocytes prior to and after IVF and following IVC.

Together, our data show that aberrations existed in the morphology of some oocytes and embryos generated in these experiments that could be detected using LM and EM but not by stereomicroscopy.

Hormone Analysis

Figure 6 presents the combined results of fecal steroid metabolite analyses for progestagens in tigers ($n = 2$ for three trials) stimulated with pFSH/pLH for follicular growth and oocyte maturation. The data indicate that luteal progestagen production in response to gonadotropin stimulation and oocyte aspiration was both delayed (by 1 wk) and significantly suppressed in trials 2 (average: 14.4 ± 4.8 μg/g) and 3 (10.4 ± 7.5 μg/g) versus trial 1 (43.3 ± 3.9 μg/g).

Average fecal estrogen concentrations following aspiration (58 ng/g) did not increase above baseline values of ~66 ng/g (43.7–100.3 ng/g) in either tiger.

These data suggest that hyperstimulation was not a factor in the ovarian response; however, the porcine hormones elicited an unnatural luteal environment.

Circulating Antibody Analysis

Antibodies against pFSH or pLH were not detected in any of the tiger samples by Ouchterloney immunodiffusion. Nonspecific binding of iodinated pFSH or pLH to gamma globulin in normal feline serum was less than 4% for each

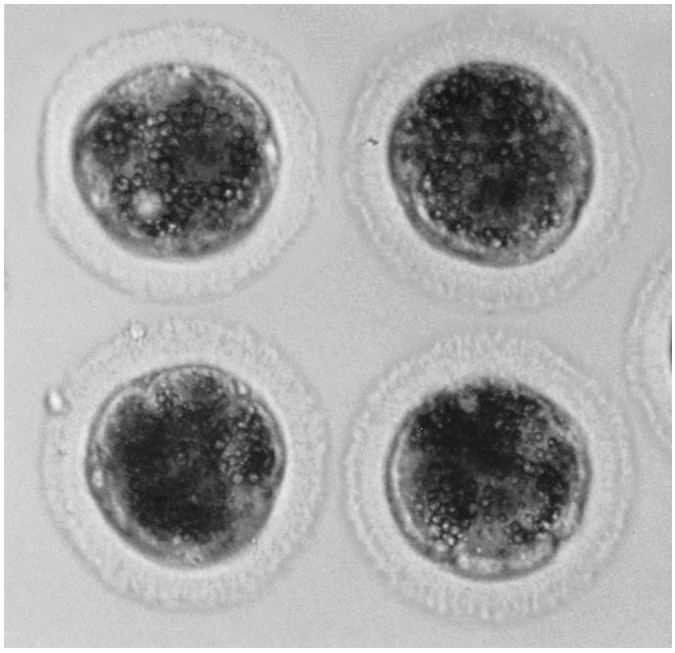

FIG. 4. Presumptive tiger embryos vitrified at the two- to four-cell stage after warming and 24-h culture.

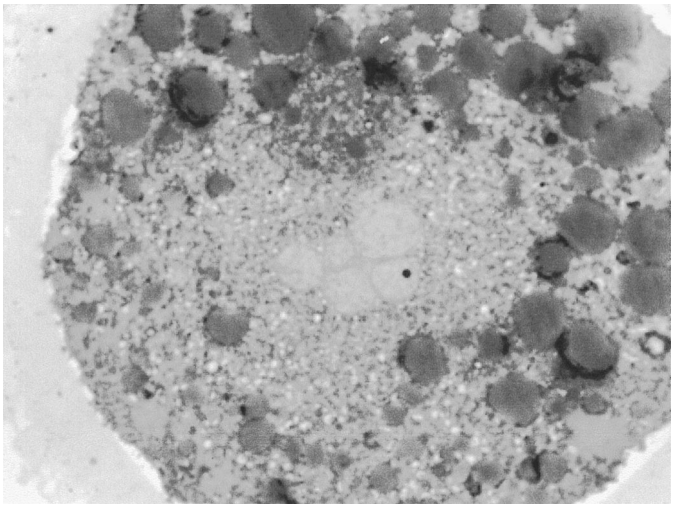

FIG. 5. Light micrograph of a polynucleated tiger zygote cultured for 24 h. Note the multiple nuclei located centrally and the large lipid droplets located peripherally (×1000). (Ultrastructure of oocyte maturation, fertilization and early embryonic development in vitro in the Siberian tiger [*Panthera tigris altaica*]. Gjorret JO, Crichton EG, Loskutoff NM, Armstrong DL, Hyttel P. Molecular Reproduction and Development 2002. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

TABLE 2. Relationship between treatment groups and nuclear oocyte maturation, fertilization, and early embryo development.*

Treatment group	Stage of maturation/fertilization/development[a]
Pre-IVF	ONII, ONII, ONBD, MI, TI, MII, MII
IVF	MI, MI, MII, PN, PN, PN, PN, NO[b]
IVC	ONBD, MI MII, 1 cell[c], 2 cell (2), 2 cell[c] (3), 4 cell, NO[b] (2)

[a] ONI, Oocyte nucleus stage I; ONII, oocyte nucleus stage II; ONBD, oocyte nucleus breakdown; MI, metaphase I; MII, metaphase II; TI, teleophase I; PN, pronucleus; NO, no nucleus or chromosomes observed.
[b] Degenerated oocyte.
[c] Polynucleated blastomeres.
* (Ultrastructure of oocyte maturation, fertilization and early embryonic development in vitro in the Siberian tiger [*Panthera tigris altaica*]. Gjorret JO, Crichton EG, Loskutoff NM, Armstrong DL, Hyttel P. Molecular Reproduction and Development 2002. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

iodinated preparation of these hormones. Binding of iodinated pFSH or pLH never exceeded nonspecific binding in any of the tiger samples or in two of the three cats previously stimulated with exogenous gonadotropins. Serum from the third treated cat showed no binding against pFSH but gave 8.4% specific binding against iodinated pLH that was reduced to 1.4% binding with 300 ng of the pLH standard preparation. Displacement of iodinated preparations with standard hormone preparations in RIAs indicated that binding sites remained intact after iodination. Together, these findings indicate that there was no immune response detected in these tigers to repeated treatment with porcine gonadotropins.

Cryopreservation of Embryos

No embryos from either controlled-rate (slow) freezing procedure resumed cleavage after warming, while 32/70 (46%) of the vitrified embryos showed evidence of further cleavage after a 24-h culture (Fig. 4). This compared with an initial cleavage rate of 55% (166/300) prior to freezing/vitrification trials. Thus, vitrification appeared to be superior to slow freezing for the cryopreservation of in vitro-produced tiger embryos.

DISCUSSION

Our initial studies indicated that the highest degree of homology with the tiger gonadotropin glycoprotein subunits was of porcine origin (98, 95, and 93% homology between common α and FSHβ and LHβ, respectively). Therefore, commercially available purified porcine gonadotropins (of improved purity over FSH-P) were targeted in this study to stimulate ovarian activity in tigers. The use of porcine gonadotropins rather than those of ovine origin was also based on a firm structural rationale. In the glycoprotein hormone α subunit, e.g., residues 24 and 25 of both porcine and tiger are Lys and Gly while the corresponding two amino acids of ovine α are Pro and Asp. A substitution of the long, charged side chain of Lys24 to an uncharged Pro residue, which, unlike Lys, allows no backbone flexibility, has the potential of altering the local conformation of the α-subunit. Furthermore, because the adjacent residue of the porcine and tiger α, Gly25, which has the most flexible side chain and the smallest, neutral side chain volume of all amino acids, is replaced by the acid residue Asp, potential immune reactivity using ovine gonadotropins is enhanced. Similarly, in the respective FSHβ subunits, the difference between the polar Thr residue in the tiger versus the hydrophobic ovine Ala at residue 50 as well as the difference between the polar Met residue in the tiger versus the hydrophobic ovine Ile at residue 109 imply that less conformational difference is likely between tiger and porcine FSH than between tiger and ovine FSH. Moreover, there are two Val to Met differences in the respective amino acid sequences of felid LHβ and ovine LHβ (at residues 52 and 78). We have previously shown that a single Val to Met alteration at codon 79 of hCG-β is sufficient to alter the activity of the human gonadotropin [61]. Taken together, these observations provide a strong justification for using porcine rather than ovine hormones for tiger stimulation.

Overall, the results indicate that ovarian responsiveness remained high when Siberian tigers were repeatedly treated with porcine hormones with no refractoriness seen. In three of four animals, the number of follicles observed at the final stimulation increased beyond the initial trial. An average of 30 oocytes (from an average of 33 follicles) retrieved/fe-

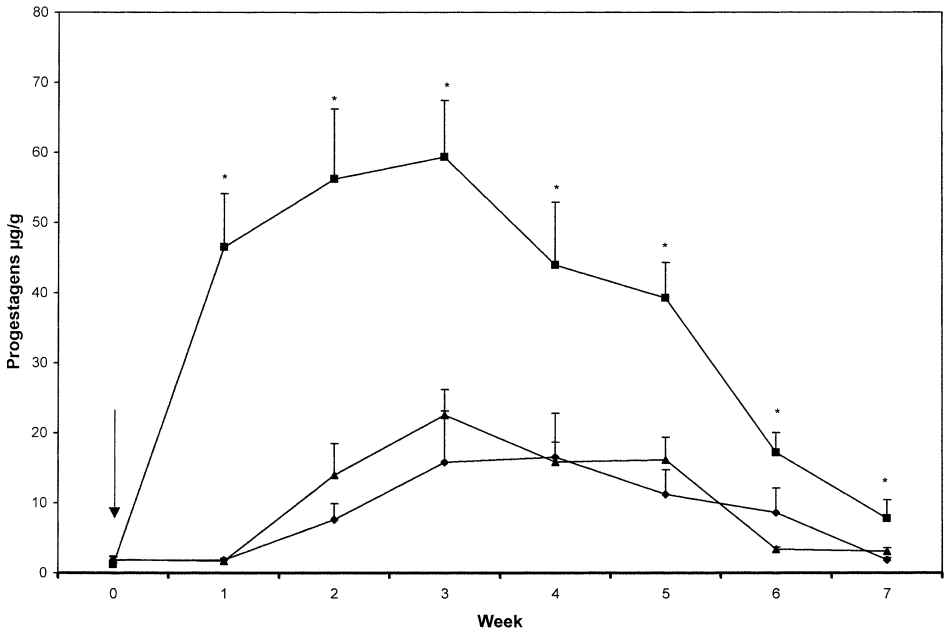

FIG. 6. Average weekly progestagen concentrations during trial 1 (squares), trial 2 (triangles), and trail 3 (diamonds) following administration of gonadotropins (arrowed). *$P < 0.05$, *t*-test comparison of trial 1 concentrations with trials 2 and 3.

male compared favorably with previous harvests by other researchers using alternative hormone regimens [9, 40]. A comparison of the number of oocytes recovered at the first versus the third oocyte retrieval procedures showed that three females produced more oocytes and one female produced half as many oocytes at the third recovery; the reason for this inconsistency is not clear. An average of 53% of the presumptive zygotes cleaved in vitro, a similar rate to that reported by Pope et al. [43], also using porcine gonadotropins; however, these rates were below a previous report in which eCG and hCG were used to stimulate ovarian activity [9] in the tiger.

Failure to detect an immunogenic response to porcine gonadotropins in an assay that tested positive in one chorionic gonadotropin-stimulated domestic cat was encouraging. It contrasts with observations of Swanson et al. [10, 29] that a high proportion of domestic felines that had been treated repetitively with eCG and hCG developed antibodies against these foreign hormones. The mounting of an immune response would mitigate against the repeated use of a hormonal ovarian stimulation regimen by promoting refractoriness over time. One potential way to eliminate this inconsistency would be to adopt a stimulation and retrieval regimen that relies on the use of homologous felid gonadotropins. Now that the sequences of these homologous gonadotropins are known (see Fig. 1), this approach becomes increasingly feasible.

While gross morphological observations of the ovarian response and recovered oocytes in our study were encouraging, it was apparent that the luteal profiles of treated tigers deviated markedly from those of naturally mated animals [62]. This was especially apparent at the second and subsequent stimulations, when analyses of fecal steroid metabolites indicated that the repeated administration of porcine gonadotropins resulted in decreased progestagen levels following oocyte aspiration. Fecal progestagen concentrations in trial 1 were similar to those observed in tigers following the administration of 1000 IU eCG and 750 IU hCG [27], but they were substantially below these values following trials 2 and 3. Further, in comparison with naturally estrual/mated animals, in which fecal progestagen concentrations averaged 34.6 μg/g [27], trial 1 levels were elevated and they fell below this value after subsequent stimulations. That the endocrine environment elicited by porcine hormones may not be physiological is not necessarily adverse because reduced progestagen levels can always be supplemented. Estrogen profiles were more encouraging. Neither of two tigers of which hormone levels were assayed showed estradiol elevations above baseline following gonadotropic stimulation. This contrasts with naturally bred (82.5 ng/g) and chorionic gonadotropic-stimulated tigers (251.7 ng/g for 15 days following hCG injection) [62] and suggests that hyperstimulation was not a factor in the response.

On examining the ultrastructure of COCs, it became apparent that their gross morphology was an inadequate predictor of maturational status, fertilizability, and developmental potential of the enclosed oocyte because some of the presumptively matured COCs contained oocytes that had failed to acquire full meiotic competence. Likewise, electron microscopy revealed that a large proportion of embryos that appeared to be normal stereomicroscopically were actually carrying aberrations such as nuclear or cellular fragmentation or polynucleation that could ultimately bring about their demise. These data are discussed in detail elsewhere [63]. While ultrastructural features have not previously been examined following the administration of exogenous gonadotropins to nondomestic felids, abnormalities in oocytes [37, 64–66], embryos [67, 68], or pregnancies [69] have resulted from such procedures in several laboratory and domestic species. Roth et al. [6], in their study of the effects of eCG and hCG on oocyte and embryo production in the cat, compared naturally estrual with stimulated animals and concluded that some factor other than either gonadotropin was responsible for the quality of embryos produced and the low pregnancy success. Furthermore, abnormal morphology of embryos does not necessarily preclude normal development.

Results obtained from cryopreservation experiments suggested that vitrification was superior to slow freezing methods for the successful cryopreservation of in vitro-produced tiger embryos. While vitrified embryos resumed development at a rate similar to that of nonfrozen embryos, none of those prepared by slow freezing methods resumed cleavage. These findings contrast with results obtained by Pope et al. [55] in which slow freezing methods met with success in cat embryos. Pushett [70] has shown vitrification to be superior to slow freezing of 9- to 16-cell cat embryos. In contrast, studies in our laboratory [71] have shown no statistically significant differences in the resumption of cleavage of in vitro-matured domestic cat embryos frozen at the 2- to 4-cell stage by fast versus slow methods. While these findings caution the direct application of methods from model species (domestic cat) to closely related counterparts (e.g., tigers), conclusions drawn from the experiments described herein are limited by our relative lack of success in generating a high percentage of quality oocytes and embryos by the ovarian stimulation methods utilized.

In conclusion, this research endorses ongoing trials in the use of purified porcine gonadotropins for ovarian stimulation in Siberian tigers, albeit with revised regimens for their delivery (e.g., [23, 72–74]). Yet the question still arises as to whether porcine gonadotropins are ultimately the optimal reagents to be using. Ongoing research in our laboratory designed to apply our knowledge of tiger FSH and LH sequences to the cloning of these gonadotropins provides hope that any potential conformational differences in the hormones used in assisted reproductive technology studies will be minimized and efforts to assist reproduction in endangered felid species optimized.

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